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Analysis of H5N1 avian influenza infections from wild bird surveillance in Hong Kong from January 2006 to October 2007

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Summary

Intensive surveillance of dead wild birds for H5N1 avian influenza infection is conducted in Hong Kong. Between January 2006 and October 2007 pooled cloacal and tracheal (C&T) swabs from 17,592 dead wild birds (from 16 different orders including 82 genera) were tested and 33 H5N1 HPAI viruses were isolated. No H5N1 infection has occurred in poultry farms or live poultry markets in Hong Kong since January 2003. The gross and histopathology in the various H5N1 infected avian species is described, along with the performance of the virology, PCR and antigen detection tests used. This evaluation also included determination of virus titres and detection limits for the H5 haemagglutinin gene (H5) and matrix gene (M) real-time reverse transcription PCR tests (RRT-PCR) in C&T swabs from 12 wild birds. The viruses isolated belonged to Clades 2.3.2 and 2.3.4 and within Clade 2.3.4 some clustering was evident based on H5 HA sequencing. However there were no differences in the pathology findings between these sub-groupings and the various diagnostic tests gave similar results for these viruses, except for a loss in sensitivity of the H5 RRT-PCR for several viruses in one cluster from birds submitted in February 2007.

Introduction

There are many subtypes of influenza A viruses based on their haemagglutinin (16 H subtypes) and their neuraminidase antigens (9 N subtypes). All H and N subtypes have been found in wild birds from at least nine different orders but the main reservoirs were believed to be birds belonging to the orders *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns, surfbirds, sandpipers) and natural infections in these species were usually asymptomatic (Swayne & Suarez, 2000). Prior to 2002 highly virulent viruses able to cause severe acute avian influenza outbreaks was generally associated with the H5, H7 or rarely certain H10 subtypes infecting chickens and other gallinaceous poultry (Alexander, 2004; Sims et al., 2005), with the exception of an outbreak of H5N3 avian influenza in wild Common Terns (*Sterna hirundo*) in South Africa in 1961 in which approximately 1,300 of

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these birds died (Becker, 1966) and outbreaks in farmed geese in Guangdong, China in 1996 (Xu et al., 1999).

Before December 2002 avian influenza viruses had not caused substantial mortalities in waterfowl or wild birds in Hong Kong although outbreaks of H5N1 highly pathogenic avian influenza (HPAI) had caused high mortality outbreaks in chickens and other gallinaceous poultry in 1997, 2001, twice in 2002 and 2003 (Sims et al, 2003, Ellis et al, 2004). In December 2002 in Hong Kong two separate outbreaks of H5N1 avian influenza with high mortality rates in waterfowl occurred in recreation parks with deaths in other wild bird species [Greater Flamingo (*Phoenicopterus ruber*), Little Egret (*Egretta garzetta*), Grey Heron (*Ardea cinerea*), a Black-headed Gull (*Larus ridibundus*), a feral pigeon (*Columba livia*) and a tree sparrow (*Passer montanus*)] (Ellis et al., 2004; Sturm-Ramirez et al., 2004). Two separate genotypes of H5N1 HPAI viruses, the Y and Z genotype were involved in these outbreaks and the Z genotype also caused infections in terrestrial poultry in some live poultry markets and 5 chicken farms in Hong Kong in late December 2002 to January 2003 (Guan et al., 2004).

Monitoring and surveillance of the poultry industry in Hong Kong has shown absence of H5N1 infection in farms and live bird markets since universal vaccination of terrestrial poultry on farms supplying the live bird markets was introduced in 2003. Since the waterfowl outbreaks and wild bird cases in late 2002, enhanced surveillance of wild birds and waterfowl has been introduced in Hong Kong with 24,787 cloacal or faecal dropping swabs from live birds and 1993 cloacal or pooled cloacal and tracheal swabs from dead wild birds being tested by virus culture in chicken embryos in 2003 to 2005. No H5N1 viruses were isolated from healthy wild birds or waterfowl in Hong Kong from 2003 to the present but H5N1 viruses were sporadically isolated from individual sick or dead wild birds [Peregrine Falcon (*Falco peregrinus*) - March 2003, Peregrine Falcon (*Falco peregrinus*) - January 2004, Grey Heron (*Ardea cinerea*) – November 2004, Grey Heron (*Ardea cinerea*) – December 2004, Chinese Pond Heron (*Ardeola bacchus*) – January 2005] in Hong Kong between 2003 and December 2005 (OIE, 2007).

Since the large outbreak of H5N1 avian influenza in wild waterfowl in Qinghai Lake, China in May 2005 (Chen et al., 2005) and the global spread of this strain of H5N1 virus (Capua and Alexander, 2007) surveillance of dead wild birds in Hong Kong was greatly increased, with resources provided by the Hong Kong SAR Government to collect and test all dead wild birds found in Hong Kong for evidence of H5N1 avian influenza infection. This surveillance resulted in the detection of H5N1 viruses in 15 wild birds in 2006 and 18 wild birds in 2007 up to the end of October 2007. This paper reports on the epidemiological, pathological and virological findings in the H5N1 infected wild birds in Hong Kong from January 2006 to October 2007.

Materials and Methods

Dead wild bird surveillance in Hong Kong

After the H5N1 outbreaks in wild birds in Qinghai Lake and subsequent wild bird cases in Europe in 2005, the Hong Kong Government commenced a program of intensive

investigation of deaths in wild birds as part of their H5N1 avian influenza surveillance program. Inspection teams from the Agriculture Fisheries and Conservation Department (AFCD) were deployed to collect dead wild birds in response to reports from the public, municipal staff and staff from housing estates, various recreational parks and the AFCD's Country Parks Branch. Public educational broadcasting encouraged the reporting of sick or dead birds through a government telephone hotline number that connected these calls to an Avian Influenza Control Desk. The AI Control Desk coordinated collection of bird carcasses and submission of carcasses that were not too decomposed to the AFCD's Tai Lung Veterinary Laboratory for testing. Carcasses were kept chilled or if not able to be tested immediately were kept frozen (at -18 to -20°C) until submitted for testing. Reporting of virological and pathological testing on the dead birds was through the AI Control Desk which collated the data on date, species, location of the carcass and test results for further analysis and reporting.

Investigation procedures

On submission of dead wild birds to the laboratory cloacal and tracheal swabs, or segments of tracheal tissue from very small birds, was collected into virus transport medium ((VTM – Medium 199 plus 2002 U/ml penicillin G, 1498 U/ml streptomycin, 1578 U/ml polymyxin B, 0.2 mg/ml sulfamethoxazole, 51.3 USP U/ml nystatin, 0.05 mg/ml gentamycin and 0.02 mg/ml ofloxacin antibiotics) and tested as a pooled cloacal and tracheal swab sample (C&T swab). For logistical reasons, especially at times with heavy sample submissions ($>100/\text{day}$), C&T swabs from up to five small birds or from three larger birds of the same species and from the same location and time were pooled. The carcasses were then stored at 4°C pending the results of the PCR testing. Swabs were tested on the same day by RRT-PCR tests for presence of H5 haemagglutinin (H5) and influenza A matrix (M) genes. Virus isolation procedures in chicken embryos were also conducted on these swabs as described below. On detection of a H5 RRT-PCR positive, the bird was subjected to a full post mortem examination. In the case of a positive from a pooled sample the individual birds in the pool were tested individually by RRT-PCR for H5 and M genes before full post mortem examination.

In the post mortem examination a repeat pooled C&T swab was taken initially and then the carcass and internal organs were examined. A range of fresh tissues was collected and submitted for immunoperoxidase staining, virological examination, bacteriological examination as appropriate and a selection of tissues including those with gross pathological changes was submitted in 10% neutral buffered formalin for histopathological examination as described previously (Ellis *et al.*, 2004). Fixed tissues were trimmed and paraffin-embedded, sectioned at $5\mu\text{m}$ and stained with haematoxylin and eosin (H&E) for histopathological examination. Cryostat sections of brain, lung and in some birds, kidney, heart, spleen and pancreas were prepared and examined after immunoperoxidase staining for influenza A nucleoprotein (NP) and avian influenza H5 haemagglutinin (H5) antigens. Repeat C&T swabs and fresh tissues collected at post mortem examination were tested by RRT-PCR for H5 and M genes and by virus isolation in chicken embryos. Fresh tissues from selected cases were cultured aerobically and anaerobically on sheep blood agar for

detection of bacterial infections and also on selective agar and broth for detection of *Mycoplasma* spp. using standard procedures.

As the H5N1 positive birds were collected at varying intervals after death and in most cases had been frozen and thawed before necropsy there was a variable degree of autolysis and freeze-thaw artifact in the carcasses. From the gross and histological changes present in the colour, consistency and microscopic architecture of various organs, a subjective score was recorded from negative (–) to 4+ depending on the level of severity of autolysis and freeze-thaw artifact in each of the birds and these scores are shown in Table 1.

Virus culture

Virus culture by allantoic cavity inoculation of 9–11 day-old SPF chicken embryos was conducted by standard procedures (Alexander, 2004). HA positive allantoic fluid (AF) was initially tested by haemagglutination inhibition (HI) tests using reference antisera (Veterinary Laboratory Agency, Weybridge and USDA, Ames) to avian influenza subtypes H5 and H9 and Newcastle disease virus by standard procedures (Alexander, 2004). Any HA positive AF that was negative in HI tests for H5, H9 and NDV viruses was tested as above against reference sera to other avian influenza and avian paramyxovirus subtypes. HA negative AFs from the first passage in eggs were re-passaged as above. HA positive AF was also tested by RRT-PCR test for H5 genome and M gene and conventional RT-PCR tests with appropriate primers were conducted on isolates to identify the N1 neuraminidase gene using the procedures below. Virus titers were expressed as Log₁₀ 50% chicken embryo infectious doses (EID₅₀) /0.1 ml.

Real-time RT-PCR test for avian influenza H5 genome

The RRT-PCR test for detection of H5 haemagglutinin gene and M gene was conducted by the method described by Spackman *et al.* (2002). The RRT-PCR used the primer sets and hydrolysis probes for H5-HA and M gene provided by Dr David Suarez (Southeast Poultry Research Laboratory, USDA, Athens, Georgia) (SEPRL primers). With some of the H5N1 viruses isolated in 2007 the original H5 primer/probe sets above lacked sensitivity, so alternative Eurasia H5 primer/probe sets provided by Dr Erica Spackman, SEPRL (EA primers) and Dr Hans Heine, Australian Animal Health Laboratory, CSIRO, Geelong, Australia (CSIRO primers) were used in the RRT-PCR test.

Conventional RT-PCR for N1 gene detection

The RT-PCR test for detection of N1 neuraminidase gene was conducted by the method described by Guan *et al.* (2002) using the primer set provided by Dr Yi Guan of the Department of Microbiology, The University of Hong Kong, Hong Kong.

Immunoperoxidase staining of viral antigens

Specimens of fresh lung, brain and other tissue were processed for cryostat sectioning and stained using an immunoperoxidase staining procedure (Ellis *et al.*, 1983) with monoclonal antibodies to influenza A nucleoprotein antigen (NP) (9010-04-1335 -supplied by Dr Peter Hooper, CSIRO Australian Animal Health Laboratories, Geelong, Australia) and avian influenza H5 haemagglutinin (MAb CP62 + CP364– these are monoclonal antibodies

produced to A/Chick/Pennsylvania/1370/83 (H5N2) haemagglutinin from St. Jude Children's Research Hospital, Memphis, Tennessee) and horseradish peroxidase anti-mouse Ig conjugate (Dako Envision™, Dako Corporation, Carpinteria, CA). Immunoperoxidase staining reactions for both H5 and NP antigens were recorded as negative, weak positive (+/-), positive (1+ to 4+) depending on extent and intensity of staining of tissues as determined by a consensus of three or four veterinary pathologists reading the tests. The results from this test were available on the same day as the post mortem examination was conducted.

Genetic characterization of the virus isolates

Further genetic characterization of the isolated H5N1 viruses was undertaken at the Centre for Emerging Infectious Diseases at the Department of Microbiology, The University of Hong Kong. This characterization included partial sequencing of the H5 HA gene over the HA1-HA2 cleavage site to establish whether they had the multi-basic amino acids characteristic of highly pathogenic avian influenza viruses, partial sequencing of the N1 neuraminidase gene and all 6 internal genes using procedures described previously (Guan *et al.*, 2002). The detailed genetic analysis of these viruses will be published separately (Guan and Peiris *et al.*, - submitted for publication)

Comparison of virus titer, virus genome and antigen load between isolates

The C&T swabs for 12 viruses detected from wild birds in 2006 and 2007 and representative of Clade 2.3.2 and the separate clusters within Clade 2.3.4 (Guan and Peiris *et al.*, submitted for publication), were titrated to determine their infectious virus titer, the genome detection limit by serial 10-fold dilution for the RRT-PCR and to determine if influenza A antigen detection tests were positive. These swabs were from the birds within each cluster that had the least evidence of autolysis or freeze-thaw changes. Ten-fold dilutions of the swab supernatant in VTM were tested by chicken embryo inoculation and RRT-PCR procedures for H5 and M genes as described above.

Influenza A antigen detection immunoassays

Comparison of the level of influenza A antigen in the pooled C&T swab for each of the 12 virus isolates tested was conducted using two influenza A nucleoprotein antigen detection immunoassays: Directigen™ Flu A kit (Becton Dickinson Microbiology Systems, Cockeysville, MD), a membrane immunoassay and Rocheby Avian Influenza Virus Antigen Test Kit (Rocheby biomed (Singapore) Pte Ltd, Singapore) a chromatographic immunoassay, using the standard protocol for these tests. The original T&C swabs had all been stored at -80°C and the antigen detection tests were done at the same time the swabs were thawed to determine their infectious virus titer by chicken embryo inoculation and virus genome load by RRT-PCR testing. As the volume remaining in the swab vials was limited they were all diluted with an equal quantity of VTM for testing by the antigen detection tests. The positive readings were graded +/- to 3+ (weakly positive to strongly positive) according to the intensity of the positive triangle or band respectively.

Results

Initial investigation data on dead wild birds

The monthly summaries of dead wild bird submitted to AFCD for avian influenza surveillance with the breakdown of families and species of birds examined from January 2006 to October 2007 is shown in Table 1 along with the details of the H5N1 virus infected birds detected. During this period pooled C&T swabs from 17,592 dead wild birds from 16 different orders of birds, including 82 genera, were tested to detect avian influenza viruses generally and H5N1 HPAI viruses specifically, and 33 H5N1 HPAI viruses were isolated. Two peaks of H5N1 virus detection in dead wild birds occurred in the winter months of 2006 and 2007 respectively but in 2007 additional isolations were made in the early summer months.

Pathology findings

The infected birds were generally in from fair to good body condition except for one young house crow (#22) but there were varying degrees of autolytic change up to severe autolysis, as shown in Table 2. Most birds (29/33) had empty proventriculus and gizzard, a Common Magpie (# 8) and a Red-billed Starling (# 30) had scanty contents and a White-backed Munia (#20) and a Peregrine falcon (#21) had a moderate quantity of ingesta present. The most common gross pathology finding was lung congestion, in 24 of 33 birds (72.7%) with lung oedema evident grossly in 16 birds (48.5%) and lung haemorrhages in only 4 birds (12.1%). Other gross changes included congestion of liver, spleen or pancreas in 8 birds (24.2%), brain congestion in 8 birds (24.2%), small focal skull haemorrhages in 7 birds (21.2%), pancreatic necrosis or haemorrhage (birds # 3 and 8), small pale spots in liver (birds # 7 and 15) or kidney (bird # 25), airsacculitis and peritonitis (bird # 21), cloudy airsacs and increased pericardial fluid (bird #12) and ecchymotic haemorrhage in pectoral muscle and overlying skin (bird # 31). More detail of the gross pathology changes in individual wild bird species is shown in Table 2.

The results of histopathological examination of individual wild birds are shown in Table 2. Histological examination of some of these birds was difficult due to the degree of autolysis present. For the birds shown with advanced autolysis (shown as 3+ and 4+ in Table 2) only moderate to severe congestion and haemorrhage and prominent areas of necrosis could be detected in H&E stained sections. Overall histopathological examination of these wild birds showed varying combinations of the following histopathology changes: congestion of lungs was evident in 32 of 33 birds, 25 had some degree of oedema and or haemorrhage in the lungs and 11 had small foci of parabronchial necrosis or peribronchial lymphocyte necrosis; the brain showed some degree of congestion in most birds with multiple small foci of parenchymal necrosis with mild gliosis in the white and/or grey matter of 9 birds, or multifocal non-suppurative encephalitis with some vasculitis in 3 birds (birds # 4, 21 and 32); congestion of cardiac vessels in many of the birds and multiple foci of acute myocarditis with myocardial degeneration/necrosis in 9 birds; varying degrees of congestion in kidneys, liver and spleen occurred in many of the birds and in kidneys of 8 birds there were multiple small foci of tubule degeneration/necrosis, the spleen of 8 birds had multiple small foci of necrosis of lymphocytes and the liver of 10 birds showed multiple small foci of

hepatocyte necrosis; there was tracheal congestion with necrosis and loss of epithelial cell and mucus glands in 7 birds; multifocal acute necrosis in the pancreas occurred in 4 birds; and multiple small foci of lymphocyte necrosis was present in caecal tonsils of 3 birds. Individual birds also had distinct lesions indicative of other disease processes including granulomatous cholangitis with hepatic trematode infection (bird # 1), multiple foci of acute necrosis associated with bacterial embolism in liver and brain (bird # 5), mild subacute cholangiohepatitis and salpingitis (bird # 8), fibrinopurulent pericarditis (bird # 15), multifocal necro-suppurative typhilitis with embryonated parasite eggs embedded in the caecal wall (bird # 21), chronic necrotising enteritis with diptheritic membranes and flagellate protozoans (*Trichomonas* sp.) present (bird # 22) and nematode parasites tracking through the renal cortex (bird # 29).

Immunoperoxidase staining of viral antigens

Immunoperoxidase staining of cryostat sections for H5 and NP antigens from these birds consistently indicated systemic spread of the virus with positive staining for both antigens being detected in brain, lung, heart, spleen, kidney, pancreas, liver, trachea and small intestines of various birds as shown in Table 2. Only cryostat sections of lung and brain were stained in all 33 birds. Positive H5 antigen staining of varying intensity and distribution was detected in brain tissue from 28 of 33 (84.8%) birds and in lung tissue from 25 of 33 (75.8%) birds. Similarly, positive NP staining was detected in brain tissue from 29 of 33 (87.9%) birds and in lung tissue from 28 of 33 (84.8%) birds. Of the other tissues, H5 antigen was detected in 12 of 16 (75.0%) hearts and all 7 kidneys, 5 spleens, 4 livers, 3 pancreases, 2 small intestines and 1 trachea examined. NP antigen was detected in 14 of 16 (87.5%) hearts and all 7 kidneys, 5 spleens, 4 livers, 4 pancreases, 2 small intestines and 1 trachea examined.

Only three birds gave negative immunoperoxidase staining for H5 antigen in both brain and lung and two of these birds, a Scaly-breasted Munia (#16) and a Japanese White-eye (# 19) also gave negative staining for NP antigen in both brain and lung. The other bird, a Red-billed Starling (#30) gave good NP antigen staining in brain, lung, heart and kidney. Bird #16 showed advanced autolysis at necropsy but other Scaly-breasted Munia (#26 and #28) also showed advanced autolysis yet gave positive H5 and NP staining in both brain and lung. The other Japanese White-eye (#6) also showed autolysis, as or more advanced than bird #19, and it showed no H5 or NP antigen staining in brain and only minimal antigen staining for both antigens in lung.

Virology test results

Isolation of H5N1 virus was positive from pooled C&T swabs, tracheal, intestinal swabs and/or various tissues from all 33 birds as indicated in Table 3. In all cases the virus was isolated from first passage in 9–11 day-old chicken embryos with death of embryos by 48 hours. Confirmation of the isolation was by H5 specific HI test on HA positive allantoic fluids, RRT-PCR for M and H5 HA genes and conventional RT-PCR for N1 gene. This was conducted on AF from all the samples submitted for the case or on a selection of the positive AF from the case as indicated in Table 3. Genetic analysis of the H5N1 viruses isolated from the wild birds during this investigation showed they belonged in H5N1 Clades 2.3.2 and

2.3.4 and the viruses in Clade 2.3.4 could also be grouped in 7 separate clusters (Guan and Peiris et al., submitted for publication). For the viruses in Clade 2.3.2 and most clusters in Clade 2.3.4 the PCR results for H5, M and N1 genes on the AF samples were all clearly positive. However, with some viruses from Clade 2.3.4 / cluster 7 (see Table 3) isolated from birds submitted between 7 and 27 February 2007 there was some variability in the sensitivity of the H5 RRT-PCR results with the three primer/probe sets that were used for this test. Allantoic fluid harvests from two viruses in this cluster (# 23. D-07-1993 and # 25. D-07-2372) were not positive by any of the three H5 RRT-PCR primers/probe sets and AF harvests from some tissues infected with three other viruses (# 24. D-07-2065.1; #24. D-07-2065.2; and # 28. D-07-2572) was only positive by the CSIRO H5 RRT-PCR primer/probe set. The M gene RRT-PCR, N1 gene RT-PCR tests and conventional RT-PCR for H5 for all AF harvests of the viruses in this cluster were consistently positive.

PCR test results on initial swab material tested

The RRT-PCR tests for HA gene and M gene and the RT-PCR test for N1 gene were performed on submitted swabs or tissues from all 33 birds as indicated in Table 3. The M gene RRT-PCR was positive for all specimens from 32 of 32 cases tested. The H5 HA gene RRT-PCR was positive on pooled C&T swabs, tracheal, intestinal swabs and/or various tissues from 31 of 33 cases tested as indicated in Table 3. Two viruses (# 23. D-07-1993 and # 25. D-07-2372) were not detected by the H5 RRT-PCR conducted directly on swabs or tissues using all three primer/probe sets (SEPR, EA or CSIRO). These viruses belonged to Clade 2.3.4 / cluster 7. The N1 gene RT-PCR was positive for pooled C&T swabs, tracheal, intestinal swabs and/or various tissues from all 33 cases tested as shown in Table 3.

Genome characterization of the wild bird H5N1 viruses isolated

All the viruses show nucleotide sequences coding for multiple basic amino acids at the HA cleavage site consistent with HPAI viruses, a deletion of nucleotides in the neuraminidase gene consistent with Z genotype H5N1 viruses, no changes from A/Gs/Gd/1/96 (H5N1) virus at the receptor binding site and the H5 HA genes fell into H5N1 virus Clade 2.3.2 (5 viruses) and Clade 2.3.4 (28 viruses) as described by Guan and Peiris et al. (submitted for publication).

Comparison of virus titer, virus genome and antigen load between isolates

The comparison of the infectious virus titres, detection limits using H5-HA gene and M gene RRT-PCR tests and detection of influenza A antigen in C&T swabs for 12 H5N1 virus infected dead wild birds in Hong Kong in 2006–2007 is shown in Table 4. This shows considerable variation in the virus titres and RRT-PCR detection limits for viruses within Clade 2.3.2 and the clusters within Clade 2.3.4 but C&T swabs from all 12 birds were readily detectable by virus isolation and PCR tests. The Directigen membrane immunoassay for NP antigen was positive on C&T swabs from 10 of the 12 dead wild birds and the Rocheby immuno-chromatography assay for NP antigen was positive on 7 of the 12 birds.

Discussion

Hong Kong has adopted and maintained a comprehensive surveillance system aimed at early detection of incursions of H5N1 HPAI and other avian influenza viruses since a series of outbreaks in poultry farms, live poultry markets or in waterfowl in recreational parks between 1997 and early 2003 (Sims et al., 2003; Ellis et al., 2004). The introduction of biosecurity improvements for poultry farms, poultry transport, wholesale and retail live bird markets, including market rest days in wholesale and retail live bird markets, where all markets were emptied of birds, cleaned and disinfected on the same day twice monthly, together with universal H5N2 avian influenza vaccination on chickens farms in Hong Kong resulted in no further cases of H5N1 HPAI in poultry farms in Hong Kong since January 2003 (Ellis et al., 2006). After the outbreaks in waterfowl and some other wild birds in December 2002, the increased wild bird and waterfowl surveillance in Hong Kong resulted in virus culture being conducted on 24,787 swabs from live birds and 1,993 swabs from dead birds in 2003 to 2005. H5N1 HPAI virus was isolated from 5 dead wild birds during that period, as described in the introduction. After the H5N1 HPAI outbreak in wild waterfowl in Qinghai Lake in May 2005 and then the subsequent spread of this virus out of East and South-east Asia to Europe, the Middle East, Africa and South Asia the surveillance of dead wild birds in Hong Kong was further increased so that between January 2006 and October 2007 pooled C&T swabs from 17,592 dead wild birds from 16 different orders of birds, including 82 genera, were tested to detect avian influenza viruses generally and H5N1 HPAI viruses specifically.

From January 2006 to October 2007 thirty three H5N1 HPAI viruses were isolated from dead wild birds in Hong Kong with an overall prevalence of 0.2% (95% CI = 0.1 to 0.3%). For the dead bird surveillance from 2003 to 2005 the prevalence of H5N1 HPAI viruses was 0.3% (5/1993; with 95% CI = 0 to 0.5%). There is no significant difference between the proportion of H5N1 HPAI virus infected wild birds between these two periods ($\chi^2 = 0.1147$, $p > 0.05$). Although the prevalences show no differences, the range of wild bird species infected in 2006–2007 was much broader and included 11 genera of Passeriformes as well as a representative of the Ciconiiformes (Little Egret) and representatives of the Falconiiformes (two Peregrine Falcons, Crested Goshawk, Common Kestrel), compared with representatives of Ciconiiformes (two Grey Herons, Chinese Pond Heron) and Falconiiformes (two Peregrine Falcons) in 2003 to 2005. Passeriformes are generally more common in urban habitats and around poultry farms than Falconiiformes and Ciconiiformes so qualitatively this is a change that could impact on risk of incursion of H5N1 viruses in Hong Kong. It is interesting to note also that the range of dead wild birds infected with H5N1 HPAI viruses in Mainland China increased after the initial outbreak in Qinghai Lake in May 2005, affecting Anseriiformes (Bar-headed Geese and Ruddy Shelducks), Charadriiformes (Great Black-headed Gulls) and Pelicaniformes (Great Cormorants), to subsequently include Passeriformes (Magpies) from Liaoning Lake in October 2005 and April 2006; other Anseriiformes (Goosander, Widgeons), other Charadriiformes (Brown-headed Gulls), other Pelicaniformes (Common Cormorant), other Passeriformes (Crows), Ciconiiformes (Little and Great Egrets), Gruiformes (Black-necked Crane), Falconiiformes (Vultures, Hawks),

Podicipediformes (Grebes), Strigiformes (Owls) and other unspecified wild birds from Qinghai Lake and Tibet in April to May 2006 (FAO EMPRESS, 2006).

The gross and histopathology change evident in this diverse group of dead wild birds was quite variable and was complicated by the level of autolysis and freeze-thaw artifact that was inherent in the system of surveillance based on submission of dead wild bird carcasses. In cases where carcasses showed minimal autolysis and freeze-thaw artifact the gross pathology often included lung congestion, with or without obvious pulmonary oedema and some birds had visceral congestion, brain congestion and focal skull haemorrhages. Although most birds had an empty proventriculus and gizzard they were generally in fair to good body condition. The presence of fair to good body condition and prominence of lung congestion on gross pathology examination was similar to the findings in the H5N1 outbreaks in waterfowl and other wild birds in Hong Kong in late 2002 (Ellis et al., 2004). The histopathology changes were also quite variable but the most consistent findings were: lung congestion with varying degrees of oedema in most, with small foci of peribronchial lymphocyte necrosis in 33.3% of birds; brain congestion in most with multiple small foci of parenchymal necrosis and gliosis in grey and/or white matter in 27.3% of birds along with presence of vasculitis and perivascular lymphoid cuffing in another 9% of birds; and varying degrees of congestion in spleen, trachea, liver, kidney, pancreas or heart with small foci of lymphoid, epithelial or myocardial necrosis respectively in some birds. The range and prevalence of the various histopathology changes in these wild birds was also similar to the findings in the H5N1 outbreaks in waterfowl and other wild birds in Hong Kong in late 2002 (Ellis et al., 2004). There was no apparent difference in the pathological changes present in birds infected with H5N1 viruses from Clade 2.3.2 and the separate clusters within Clade 2.3.4.

Rapid immunohistochemical investigation using immunoperoxidase staining with monoclonal antibodies to H5-HA and influenza A NP antigens was conducted on cryostat sections of necropsy tissues from all the birds giving positive H5 or M RRT-PCR results. Results were generally available within 4 hours after the necropsy and this provided a rapid specificity check on the PCR positive cases. This also enabled examination of tissues for evidence of lesions consistent with HPAI infection as well as detecting the presence of and semi-quantitative assessment of the amount of viral antigens in tissues. This investigation clearly showed that these H5N1 viruses were neurotropic in a range of wild bird species and replicated in a range of tissues as well as lung in these birds. This has been a feature of H5N1 HPAI viruses in waterfowl and other wild bird species since December 2002 (Sturm-Ramirez et al., 2004; Ellis et al., 2004). As with the other pathology findings there were no apparent differences in the distribution, or quantity of viral antigens in tissues from birds infected with H5N1 viruses from Clade 2.3.2 and the separate clusters within Clade 2.3.4. These viral antigens were also able to be detected fairly consistently in birds that were showing moderate to severe autolysis. Only two H5N1 infected small birds (a Japanese White-eye and a Scaly-breasted Munia) showed no evidence of viral antigen staining in the cryostat tissue sections examined and one other H5N1 infected bird (Red-billed Starling) showed no staining of H5-HA antigens in brain or lung but moderate staining for NP antigens in these tissues.

From all 33 affected dead wild birds H5N1 viruses were isolated by SPF chicken embryo allantoic cavity inoculation of C&T swabs, or trachea or lung swabs in two birds that did not have C&T swabs collected, and from a range of other tissues from these birds indicated in Table 3. In all cases presence of H5 virus isolation was confirmed by H5 specific HI testing of HA positive allantoic fluid harvests and N1 was confirmed by N1 specific RT-PCR tests on the original swabs and in some birds on the allantoic fluid harvests. This included a number of birds, as shown in Table 2, which were showing advanced signs of autolysis or freeze-thaw artifact. For the H5N1 viruses from Clades 2.3.2 and 2.3.4, the M specific RRT-PCR showed complete agreement and for Clades 2.3.2 and most clusters from Clade 2.3.4, the H5 specific RRT-PCR showed complete agreement with the virus isolation results for the original swabs or tissues and allantoic fluid harvests after culture of these swabs or tissues. However, in one cluster of Clade 2.3.4 viruses (cluster 7) there was a reduction in sensitivity of the H5 specific RRT-PCR, with two of nine viruses in the cluster isolated in February 2007 not detected in the original swabs or allantoic fluid harvests by any of the three primer/probe sets being used. Three other viruses in this cluster isolated at the same time (2 x Silver-eared Mesia and one Scaly-breasted Munia) were only detected in allantoic fluid harvests by the CSIRO primer/probe set.

There was considerable variation in the H5N1 virus titre ($10^{1.0}$ to $10^{6.3}$ EID₅₀/0.1ml) being shed in C&T swabs collected from 12 affected birds that represented viruses from Clades 2.3.2 and separate clusters from Clade 2.3.4, and were showing the least degree of autolysis or freeze-thaw artifact. This included all 5 viruses from (Clade 2.3.2) and a representative from each cluster in Clade 2.3.4. Similarly, there was considerable variation in the detection limit by H5 RRT-PCR for these specimens, from a dilution of 1:10 to 1: 10^5 and for some viruses the correlation between virus titre and PCR detection limit was poor. With the M RRT-PCR the detection limit was consistently lower (undiluted to 1:10 dilution) than that for the H5 RRT-PCR and the virus titre and the relationship between the M RRT-PCR detection limit and the virus titre was more consistent. Despite the wide variation in virus titres and PCR detection limits it was encouraging from a field diagnosis perspective that all the dead H5N1 infected wild birds were readily detected by virus culture and M RRT-PCR tests and 31 of 33 were detected by H5 RRT-PCR on initial C&T, tracheal or lung swabs.

In considering the reasons for the lower sensitivity for the H5 RRT-PCR for some viruses in cluster 7 of Clade 2.3.4 it is possible that there may have been insufficient intact H5 RNA template in the sample or that there were significant mismatches between the primers/probes and the target sequence on the H5 HA gene for these viruses. The virus from Clade 2.3.4/cluster 7 included in the detection limit study (#22. D-07-1203) had a virus titre in C&T swabs of $10^{5.3}$ EID₅₀/0.1ml compared with H5 PCR detection limit of 1: 10^3 , so if the problem viruses were only present at a titre of 10^2 EID₅₀/0.1ml it would be possible the H5 PCR test was below the detection limit. However when this virus was present at a titre $10^{5.3}$ EID₅₀/0.1ml, the M RRT-PCR test was only positive on the undiluted sample. If the virus was only present at a titre of 10^2 EID₅₀/0.1ml the M RRT-PCR could also be expected to be below the detection limit. However, all viruses in this cluster with negative or variable H5 RRT-PCR results were all clearly positive by M RRT-PCR. It is more likely that the poorer sensitivity for the H5 RRT-PCR test for these viruses resulted from mismatches between target RNA and primer/probe sequence and this is being investigated further.

Although the sensitivity of the rapid antigen tests for influenza A NP antigen on C&T swabs from the dead wild birds were lower than by virus isolation and RRT-PCR tests the sensitivity for the Directigen test (83.3% with 95% CI = 62.2–100%) was higher than the range of the sensitivities for 5 rapid antigen detection tests (including the Rockyby test) for H5N1 avian influenza on swabs from dead waterfowl and wild birds in Hong Kong collected in 2002, which ranged from 29.3% (95% CI = 19.0–36.6%) to 50.7% (95% CI = 39.4–62.0%) (Chua et al., 2007). The sensitivity of the Rockyby test in the current study (58.3% with 95% CI = 30.4–86.2%) was at least as sensitive as it had been in the previous study (30.7% with 95% CI = 20.3–41.1%). With this level of sensitivity and the simplicity of these rapid antigen detection tests there is potential for them to be used for testing swabs from dead wild birds as part of a wild bird H5N1 avian influenza surveillance programs in remote locations. This would provide preliminary information about presence of influenza A antigens in dead wild birds. However, this would require protocols to be developed to ensure that testing was conducted on the freshest specimens available, that several dead birds from the site were tested and that appropriately preserved specimens were forwarded as soon as possible for confirmatory virus testing by PCR or virus culture procedures.

In conclusion, the intensive surveillance program based on virological testing of dead wild birds in Hong Kong has continued to identify that incursions of H5N1 HPAI do occur in wild birds, albeit at a low prevalence, but this continues to reinforce the approach taken by the administration and the poultry farmers in Hong Kong to maintain a high level of farm and market biosecurity and an ongoing H5 avian influenza vaccination program. This approach has resulted in the absence of H5N1 HPAI from poultry farms in Hong Kong since January 2003 and there has been only one incursion of H5N1 virus into live poultry markets in June 2008. The dead wild bird surveillance program has contributed detailed information about the prevalence, pathology and molecular epidemiology of H5N1 HPAI viruses in a wide variety of wild avian species and provided an evaluation of the diagnostic virology procedures used for detection and confirmation of H5N1 HPAI in wild avian species. Although the viruses could be separated into different clades and clusters based on sequence analysis of the H5 HA genes no differences were evident in their pathological properties in wild birds. Minor genetic variation in some viruses from one cluster in Clade 2.3.4 resulted in some loss of sensitivity of the H5 RRT-PCR test but this did not affect other molecular diagnostic or virology tests on these viruses.

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Table 1

Wild bird surveillance summary showing information about the birds tested and H5N1 viruses isolated

Month	Order (numbers and common names)	Total tested	H5N1 Virus isolated	Common name of bird	Location
January 2006	Anseriformes (1 - duck), Charadriiformes (3 - Eurasian woodcock), Ciconiiformes (11 - egrets, herons), Columbiformes (139 - doves, pigeons), Coraciiformes (2 - dollabirds, kingfishers), Falconiformes (5 - buzzard, goshawk, sparrowhawk), Galliformes (7 - francolin, pheasant, quail), Gruiformes (3 - waterhens), Passeriformes (324 - *), Psittaciformes (17 - budgerigars, cockatiel, lovebirds, parrots), Strigiformes (2 - owls), Unidentified (9)	523	1. D-06-0075 2. D-06-0366 3. D-06-0540	Magpie Robin Magpie Robin Crested Myna	Tai Po Sha Tau Kok Wong Tai Sin
February 2006	Anseriformes (4 - ducks, goose), Apodiformes (6 - swifts), Charadriiformes (4 - black-headed gull, Eurasian woodcocks, marsh sandpiper), Ciconiiformes (13 - egrets, herons), Columbiformes (828 - doves, pigeons), Coraciiformes (6 - dollabirds, kingfishers), Cuculiformes (11 - coucal, cuckoo, koel), Falconiformes (6 - buzzard, goshawk, kestrel), Galliformes (15 - chicken, francolin, pheasant, quail), Gruiformes (1 - waterhens), Passeriformes (1594 - *), Pelicaniformes (1 - cormorant), Psittaciformes (59 - budgerigars, cockatiel, lovebirds, parrots), Strigiformes (9 - owls), Unidentified (20)	2577	4. D-06-0645 5. D-06-0718 6. D-06-1038 7. D-06-2125 8. D-06-2256 9. D-06-2454 10. D-06-2469 11. D-06-2512 12. D-06-2648 13. D-06-2858 14. D-06-3033	Common Magpie Little Egret Japanese White Eye Common Magpie Common Magpie Munia White-backed Munia Large-billed Crow House Crow House Crow Common Magpie	Sham Tseng Tuen Mun Mongkok Sham Shui Po Mongkok Repulse Bay Wanchai Sham Shui Po Cheung Sha Wan Shek Kip Mei Shouson Hill
March 2006	Anseriformes (1 - duck), Apodiformes (23 - swifts), Caprimulgiformes (1 - nightjar), Charadriiformes (4 - Eurasian woodcock, sanderling), Ciconiiformes (18 - egrets, herons), Columbiformes (770 - doves, pigeons), Coraciiformes (8 - kingfishers), Cuculiformes (18 - coucal, cuckoo, koel), Falconiformes (10 - buzzard, falcon, goshawk, kestrel, sparrowhawk), Galliformes (3 - chicken, tragopan), Gruiformes (4 - crane, waterhens), Passeriformes (1607 - *), Pelicaniformes (2 - cormorant), Psittaciformes (39 - budgerigars, cockatiel, lovebirds, parrots), Strigiformes (1 - owls), Unidentified (48)	2542	15. D-06-5211	Peregrine Falcon	Tin Shui Wai
April 2006	Anseriformes (1 - duck), Apodiformes (11 - swifts), Caprimulgiformes (3 - nightjar), Ciconiiformes (17 - egrets, herons), Columbiformes (496 - doves, pigeons), Coraciiformes (3 - kingfishers), Cuculiformes (37 - coucal, cuckoo, koel), Falconiformes (8 - buzzard, goshawk, black kite), Galliformes (26 - chicken, francolin, pheasant, quail, tragopan), Gruiformes (4 - crane, moorhen, waterhens), Passeriformes (858 - *), Psittaciformes (15 - budgerigars, cockatiel, lorikeet, lovebirds, parrots), Strigiformes (1 - owls), Unidentified (45)	1530			
May 2006	Apodiformes (2 - swifts), Caprimulgiformes (1 - nightjar), Ciconiiformes (14 - egrets, herons), Columbiformes (253 - doves, pigeons), Coraciiformes (1 - kingfishers), Cuculiformes (26 - coucal, cuckoo, koel), Falconiformes (2 - buzzard, black kite), Galliformes (4 - chicken, pheasant), Gruiformes (3 - crane, moorhen, waterhens), Passeriformes (561 - *), Psittaciformes (3 - budgerigar, lovebird, parrot), Strigiformes (1 - owls), Unidentified (10)	883			
June 2006	Apodiformes (6 - swifts), Ciconiiformes (11 - egrets, herons), Columbiformes (63 - doves, pigeons), Coraciiformes (2 - kingfishers), Cuculiformes (3 - koel), Falconiformes (4 - buzzard, falcon), Galliformes (3 - chickens, tragopan), Gruiformes (4 - watercock, waterhen), Passeriformes (244 - *), Psittaciformes (6 - *, cockatiel, lovebirds, parrots), Unidentified (5)	349			

Month	Order (numbers and common names)	Total tested	H5N1Virus isolated	Common name of bird	Location
July 2006	Apodiformes (1 – swift), Ciconiiformes (6 – egrets, herons), Columbiformes (28 – doves, pigeons), Coraciiformes (3 – kingfishers), Cuculiformes (3 – coucals, cuckoo), Galliformes (2 – francolin, pheasant), Gruiformes (3 – crane, waterhens), Passeriformes (150 – *), Psittaciformes (3 – budgerigar, cockatiel, parakeet,). Unidentified (4)	203			
August 2006	Apodiformes (3 – swifts), Caprimulgiformes (1 – nightjar), Ciconiiformes (5 – egrets, herons), Columbiformes (22 – doves, pigeons), Coraciiformes (9 – kingfishers), Cuculiformes (3 – coucals, koel), Galliformes (2 – chicken, pheasant), Passeriformes (140 – *), Psittaciformes (1 – cockatiel), Strigiformes (1 – owl), Unidentified (1)	188			
September 2006	Anseriformes (1 – duck), Apodiformes (25 – swifts), Charadriiformes (2 – phalarope, tern), Ciconiiformes (8 – bittern, egrets, herons), Columbiformes (45 – doves, pigeons), Coraciiformes (6 – kingfishers), Cuculiformes (9 – coucals, cuckoo, koels), Falconiformes (3 – buzzard, eagle, goshawk), Galliformes (6 – chicken, pheasants, quails), Gruiformes (4 – cranes, rail, waterhens, watercocks), Passeriformes (168 – *), Psittaciformes (2 – cockatiel, parrot), Strigiformes (1 – owl), Unidentified (1)	287			
October 2006	Charadriiformes (8 – jacana, woodcock), Ciconiiformes (13 – bittern, egrets, herons), Columbiformes (59 – doves, pigeons), Coraciiformes (3 – kingfishers), Cuculiformes (14 – coucals, koels), Falconiformes (3 – besra, hobby, sparrowhawk), Galliformes (5 – chicken, quails), Gruiformes (33 – cranes, waterhens, watercocks), Passeriformes (199 – *), Psittaciformes (2 – lovebird, parrot), Unidentified (4)	343			
November 2006	Anseriformes (25 – pintails, teal and wigeon ducks), Apodiformes (2 – swifts), Caprimulgiformes (1 – nightjar), Charadriiformes (6 – snipe, woodcock), Ciconiiformes (8 – bittern, egrets, herons), Columbiformes (43 – doves, pigeons), Cuculiformes (3 – coucal, koels), Falconiformes (8 – besra, buzzard, falcon, goshawk, hen harrier, shikra, sparrowhawk), Galliformes (3 – partridge, pheasants), Gruiformes (25 – cranes, moorhens, waterhens, watercocks), Passeriformes (231 – *), Unidentified (1)	356			
December 2006	Anseriformes (1 – pintail duck), Charadriiformes (3 – avocet, woodcock), Ciconiiformes (4 – egret, herons), Columbiformes (21 – doves, pigeons), Cuculiformes (2 – coucal, koel), Falconiformes (2 – buzzard, black kite) Galliformes (1 – pheasant), Gruiformes (1 – waterhen), Passeriformes (259 – *), Psittaciformes (1 – lovebird), Unidentified (5)	300			
January 2007	Anseriformes (3 – goose, shelduck, swan), Charadriiformes (3 – avocet, woodcock), Ciconiiformes (14 – egrets, herons, spoonbill), Columbiformes (304 – doves, pigeons), Coraciiformes (2 – kingfishers), Cuculiformes (7 – coucals, koels), Falconiformes (8 – buzzards, falcon, goshawks, black kite, kestrel) Galliformes (3 – chickens, pheasant), Gruiformes (6 – crane, moorhen, waterhens), Passeriformes (1354 – *), Pelicaniformes (1 – cormorant), Podicipediformes (1 – grebe), Psittaciformes (9 – budgerigars, cockatiels, cockatoo, conure, lovebird), Strigiformes (2 – owl), Unidentified (2)	1719	16. D-07-0045 17. D-07-0458 18. D-07-0719 19. D-07-0737 20. D-07-0828 21. D-07-1143 22. D-07-1203	Scaly-breasted Munia Crested Goshawk House Crow Japanese White Eye White-backed Munia Peregrine Falcon House Crow	Wanchai Sham Shui Po Sham Shui Po San Po King Mongkok Tseun Wan Sham Shui Po
February 2007	Anseriformes (9 – ducks, swan), Apodiformes (2 – swifts), Charadriiformes (5 – gulls, plover, sandpiper, woodcock), Ciconiiformes (12 – egrets, herons, spoonbill), Columbiformes (275 – doves, pigeons), Coraciiformes (2 – kingfishers), Cuculiformes (6 – coucals, koels), Falconiformes (7 – buzzards, eagle, goshawks, kestrel), Galliformes (8 – chickens, francolin, pheasants, quail), Gruiformes (2 – crane, waterhens), Passeriformes (550 – *), Pelicaniformes (1 – cormorant), Psittaciformes (10 – budgerigars, cockatiels, lovebirds), Strigiformes (4 – owl), Unidentified (1)	894	23. D-07-1993 24. D-07-2065 25. D-07-2372 26. D-07-2433 27. D-07-2442 28. D-07-2572	Blue Magpie Silver-eared Mesia x2 Common Kestrel Scaly-breasted Munia Chestnut Munia Scaly-breasted Munia	Sham Shui Po Mongkok Sham Shui Po Happy Valley Kowloon City Sham Shui Po

Month	Order (numbers and common names)	Total tested	H5N1Virus isolated	Common name of bird	Location
March 2007	Anseriformes (7 – ducks), Apodiformes (2 – swifts), Charadriiformes (2 – gull, woodcock), Ciconiiformes (6 – egrets, herons, spoonbill), Columbiformes (309 – doves, pigeons), Coraciiformes (8 – kingfishers), Cuculiformes (5 – koels), Falconiformes (6 – besra, buzzard, black kites, sparrowhawk), Galliformes (4 – pheasants), Gruiformes (1 – waterhen), Passeriformes (450 – *), Pelicaniformes (1 – cormorant), Psittaciformes (9 – budgerigars, cockatiels cockatoo, lovebirds) Unidentified (9)	819	29. D-07-2762	Long-tailed Shrike	Hung Hom
April 2007	Anseriformes (1 – duck), Apodiformes (7 – swifts), Caprimulgiformes (1 – nightjar), Charadriiformes (4 – phalaropes, sandpiper), Ciconiiformes (9 – bittern, egrets, herons), Columbiformes (269 – doves, pigeons), Coraciiformes (7 – kingfishers), Cuculiformes (25 – coucals, cuckoos, koels), Falconiformes (1 – goshawk), Galliformes (3 – chickens, pheasant), Gruiformes (6 – crakes, rail, waterhens), Passeriformes (481 – *), Psittaciformes (5 – budgerigar, cockatiels, lovebirds), Strigiformes (2 – owl), Unidentified (6)	826			
May 2007	Apodiformes (4 – swifts), Caprimulgiformes (1 – nightjar), Charadriiformes (1 – pied avocet), Ciconiiformes (10 – bittern, egrets, herons), Columbiformes (167 – doves, pigeons), Cuculiformes (20 – cuckoos, koels), Falconiformes (7 – eagles, goshawks, black kite), Galliformes (1 – francolin), Gruiformes (4 – moorhen, rail, waterhens), Passeriformes (408 – *), Psittaciformes (5 – budgerigars, cockatiels), Strigiformes (1 – owl), Unidentified (2)	632	30. D-07-4925	Red-billed Starling	Kowloon City
June 2007	Apodiformes (28 – swifts), Ciconiiformes (5 – egret, herons), Columbiformes (195 – doves, pigeons), Coraciiformes (4 – kingfishers), Cuculiformes (18 – coucal, cuckoos, koels), Falconiformes (5 – buzzard, falcon, black kites), Passeriformes (482 – *), Psittaciformes (3 – budgerigar, cockatiel, lovebird), Unidentified (2)	742	31. D-07-5052 32. D-07-5288	Common Magpie House Crow	Shatin Sham Shui Po
July 2007	Apodiformes (7 – swifts), Ciconiiformes (5 – egrets, herons), Columbiformes (173 – doves, pigeons), Coraciiformes (9 – kingfishers), Cuculiformes (10 – coucals, cuckoos, koels), Falconiformes (6 – besras, goshawks, black kite, sparrowhawk), Gruiformes (1 – waterhen), Passeriformes (445 – *), Psittaciformes (2 – budgerigar, cockatiel), , Strigiformes (1 – owl)	659			
August 2007	Apodiformes (11 – swifts), Charadriiformes (1 – sandpiper), Ciconiiformes (12 – egrets, herons), Columbiformes (109 – doves, pigeons), Coraciiformes (6 – kingfishers), Cuculiformes (9 – coucals, cuckoos, koels), Falconiformes (2 – crested goshawks), Galliformes (12 – Crakes, Moorhens, water rails, waterhens), Passeriformes (226 – *), Psittaciformes (5 – budgerigars, cockatiels, lovebirds)	384			
September 2007	Apodiformes (19 – swifts), Caprimulgiformes (1 nightjar), Charadriiformes (1 – winbrel), Ciconiiformes (18 – bitterns, egrets, herons), Columbiformes (109 – doves, pigeons), Coraciiformes (4 – kingfishers), Cuculiformes (9 – coucals, cuckoos, koels), Falconiformes (2 – crested goshawks), Galliformes (6 – quails, chickens, francolins, pheasants), Gruiformes (12 – Crakes, Moorhens, water rails, waterhens), Passeriformes (274 – *), Psittaciformes (3 – cockatiels, lovebirds)	458			
October 2007	Apodiformes (1 – swift), Charadriiformes (22 – snipes and woodcocks), Ciconiiformes (21 – bitterns, egrets, herons), Columbiformes (120 – doves, pigeons), Coraciiformes (8 – kingfishers), Cuculiformes (12 coucals, cuckoos, koels), Falconiformes (42 – buzzards, kites, sparrowhawks), Galliformes (6 – quails, pheasants, tragopans), Gruiformes (69 – crakes, moorhens, waterhens, watercocks, waterhens), Passeriformes (209 – *), Pelicaniformes (1 – cormorant), Psittaciformes (2 – budgerigars, parakeets), Strigiformes (3 – owls)	478			

* includes bulbuls, canaries, crows, drongos, finches, flycatchers, leiothrix, laughingthrushes, magpies, munias, mynas, orioles, pipits, robins, shrike, sparrows, stonechats, starlings, sunbirds, swallows, thrushes, tits, wagtails, warblers and Japanese white-eyes.

Table 2

The pathological findings, including immunoperoxidase staining for H5 and NP antigens, in H5N1 avian influenza virus infected wild birds in Hong Kong in 2006–2007

Viruses isolated Bird species	H5N1 Clade/Cluster #	Level of autolysis	Gross pathology	Histopathology (Haematoxylin and Eosin stained sections)	IPX stain H5 Mab*			IPX stain NP Mab*		
					B	L	O	B	L	O
1. D-06-0075 Magpie Robin	2.3.4 /6	2+	Lung congestion	Lung congestion and oedema with foci of peribronchial lymphocyte necrosis; granulomatous cholangitis with trematodes in bile ducts; kidney, heart, brain congestion; muscle haemorrhage	1+	2+		2+	2+	3+
2. D-06-0366 Magpie Robin	2.3.4 /6	-	Lung congestion and haemorrhage	Lung congestion, oedema and haemorrhage; congestion and multifocal necrotising tracheitis; brain, kidney gizzard and intestines congested; lymphoid depletion/lysis in spleen and Peyer's patches, midzonal vacuolar degeneration in liver; individual fibre degeneration in muscle	1+	2+		1+	2+	
3. D-06-0540 Crested Myna	2.3.4 /5	2+	Lung oedema and congestion; spleen, liver congestion; pancreatic necrosis, focal skull and brain haemorrhage	Lung congestion and some oedema; liver congestion and random small foci of necrosis; kidney, spleen, heart, brain showed marked congestion pancreas had a focal area of necrosis; some lymphocyte necrosis in random submucosal lymphoid nodules in gizzard and cloaca	+/-	2+	*1	1+	3+	*1
4. D-06-0645 Common Magpie	2.3.4 /1	-	Lungs oedema and congestion with greyish mottling; patchy reddening of pancreas	Lung congestion and oedema with anthracoid pigment present; kidney congestion and multiple small random foci of tubule necrosis; heart had several foci of myocardial necrosis; brain had congestion, foci of necrotising vasculitis and neuropile necrosis with some gliosis and perivascular lymphoid cuffing; liver and spleen were congested and had haemosiderosis; stomach, intestines and pancreas were congested	1+	2+	*2	1+	3+	*2
5. D-06-0718 Little Egret	2.3.4 /6	4+	No changes evident	Brain showed congestion and a few small foci of neuropile necrosis with mild gliosis; lung congestion	2+	-		3+	-	
6. D-06-1038 Japanese White-eye	2.3.4 /4	3+	No changes evident	Lung congestion; multiple small foci of acute necrosis in the liver with bacterial emboli in liver and brain consistent with septicaemia	-	+/-		-	1+	
7. D-06-2125 Common Magpie	2.3.4 /4	-	Trachea congested, lung oedema; 1mm white spots in liver	Lung congestion and oedema with foci of peribronchial lymphocyte necrosis; multiple foci of acute pancreatic necrosis; multiple small foci of lymphocyte necrosis in caecal tonsils; splenic congestion and foci of lymphoid lysis; brain, kidney congestion; liver had subacute cholangiohepatitis	2+	2+		3+	3+	

Viruses isolated Bird species	H5N1 Clade/Cluster #	Level of autolysis	Gross pathology	Histopathology (Haematoxylin and Eosin stained sections)	IPX stain H5 Mab*			IPX stain NP Mab*		
					B	L	O	B	L	O
8. D-06-2256 Common Magpie	2.3.4 /4	-	Lung congestion, haemorrhages in lung and pancreas	Lung congestion and oedema with anthracoid pigment present and a fluke present in bronchus; multifocal necrotising tracheitis; general visceral and brain congestion; foci of lymphocyte lysis in caecal tonsil; flukes present in ureter; mild subacute cholangiohepatitis and salpingitis	3+	3+		3+	3+	
9. D-06-2454 Munia	2.3.4 /4	3+	haemorrhage and oedema in lungs; liver congestion; skull haemorrhage	Moderate autolysis but liver, heart, kidney, lung, spleen, brain and proventriculus all showed moderate to marked congestion with no other changes evident	4+	4+		1+	4+	
10. D-06-2469 White-backed Munia	2.3.4 /4	3+	congestion and oedema in lungs; liver congestion; skull haemorrhage	Lung congestion and oedema; brain, liver and kidney congestion; heart congestion and focal epicardial haemorrhage	2+		+/-	3+	1+	
11. D-06-2512 Large-billed Crow	2.3.4 /4	1+	Lung haemorrhage, congestion, oedema; spleen and liver congestion	Lung congestion and oedema with foci of peribronchial lymphocyte necrosis; tracheal epithelial cell necrosis; general visceral and brain congestion; focal lymphoid necrosis in lymphoid tissue in spleen, liver and kidney	2+	1+	*3	2+	1+	*3
12. D-06-2648 House Crow	2.3.4 /4	-	Lung oedema; cloudy airsacs; pericardial oedema	Lung congestion with foci of peribronchial lymphocyte necrosis; acute tracheitis; splenic lymphoid necrosis; brain showed multifocal neuronal degeneration and mild gliosis; ischaemic necrosis in pancreas; liver had occasional individual cell necrosis	4+		+/-	4+	2+	*4
13. D-06-2858 House Crow	2.3.4 /4	1+	No changes evident	Lung congestion and oedema with foci of peribronchial lymphocyte necrosis; brain showed congestion and a few small foci of neuropile necrosis with mild gliosis	3+	-		3+	1+	
14. D-06-3033 Common Magpie	2.3.4 /4	1+	Lung congestion and oedema, patchy brain congestion	liver, kidney and lung very congested; spleen congested and multiple small foci of necrosis; brain and heart mildly congested	3+	1+	*5	3+	2+	*5
15. D-06-5211 Peregrine Falcon	2.3.2	-	Trachea congested, lung oedema; 1mm white spots in liver	Lung congestion and oedema; tracheal congestion; multifocal splenic necrosis; brain had marked congestion and multiple small foci of necrosis (glial cells, neurones and blood vessel walls); congestion and multiple small foci of necrosis in kidney, liver and oviducts; heart had fibrinopurulent pericarditis	4+	-		4+	-	
16. D-07-0045 Scaly-breasted Munia	2.3.4 /2	4+	No changes evident	Lung oedema and haemorrhage; general congestion in viscera and brain; multiple small foci of acute necrosis in the liver	-	-		-	-	

Viruses isolated Bird species	H5N1 Clade/Cluster #	Level of autolysis	Gross pathology	Histopathology (Haematoxylin and Eosin stained sections)	IPX stain H5 Mab*			IPX stain NP Mab*		
					B	L	O	B	L	O
17. D-07-0458 Crested Goshawk	2.3.4 /2	-	Congestion and oedema in lungs; splenic congestion; skull haemorrhage	Lung congestion and oedema with foci of peribronchial lymphocyte necrosis; multifocal splenic and pancreatic necrosis; brain congestion with multiple small foci of neuropile necrosis and mild gliosis; focus of myocardial degeneration; focal lymphoid necrosis in thymus and caecal tonsils; congestion in liver and kidneys	3+	3+	*6	3+	3+	*6
18. D-07-0719 House Crow	2.3.4 /7	2+	Lung congestion; skull haemorrhage, brain congested	Lung congestion and oedema; brain congestion with focus in cerebrum with multifocal neuronal degeneration/necrosis; liver and kidney congestion	3+	-	-	3+	-	-
19. D-07-0737 Japanese White-eye	2.3.4 /7	2+	Lung congestion	Lung congestion, oedema and haemorrhage; liver was very congested and had a focal area of hepatocyte necrosis with degenerate heterophil and mononuclear cell infiltrate; brain was mildly congested	-	-	-	-	-	-
20. D-07-0828 White-backed Munia	2.3.4 /7	2+	Trachea, lung and brain congestion	Lung congestion with some oedema and diffuse parabranchitis; brain congestion with some foci of neuropile necrosis and gliosis; liver congestion	3+	4+	4+	4+	4+	4+
21. D-07-1143 Peregrine Falcon	2.3.2	-	Lung congestion and oedema; airsacculitis and peritonitis; focal brain congestion	Lung congestion and oedema; brain showed multifocal necrotising non-suppurative encephalitis and vasculitis; multifocal necrotising pancreatitis; intestinal trematode infection; severe multifocal necro-suppurative typhlitis with embryonated parasite eggs embedded in the caecal wall; liver, trachea and kidney showed congestion and multiple small foci of necrosis; muscle, heart, spleen were congested	2+	+/-	*7	2+	+/-	*7
22. D-07-1203 House Crow	2.3.4 /7	-	Poor body condition; brain congestion	Lung congestion; multiple small foci of neuropile necrosis and gliosis in the brain; heart showed multifocal acute myocarditis; Chronic necrotising enteritis with diptheritic membrane formation and flagellate protozoans (<i>Trichomonas</i> sp.) present	2+	+/-	*8	2+	1+	*8
23. D-07-1993 Blue Magpie	2.3.4 /7	-	Moist pharyngeal lining, lung oedema	Lung congestion and oedema with small necrotic foci in parabronchi and peribronchial lymphoid tissue; necrotising airsacculitis and tracheitis; small foci of acute myocardial degeneration; small foci of necrosis in renal pelvis; multiple small foci of neuropile necrosis and gliosis in the brain; liver showed congestion and haemosiderosis; intestines had small foci of necrosis in the muscularis layer	3+	3+	*9	3+	3+	*9
24. D-07-2065.1 Silver-eared Mesia	2.3.4 /7	2+	Mildly oedematous lungs	Lung and brain congestion	1+	1+	*10	1+	2+	*10

Viruses isolated Bird species	H5N1 Clade/Cluster #	Level of autolysis	Gross pathology	Histopathology (Haematoxylin and Eosin stained sections)	IPX stain H5 Mab*			IPX stain NP Mab*		
					B	L	O	B	L	O
24. D-07-2065.2 Silver-eared Mesia	2.3.4 /7	2+	Lung congestion and oedema; liver, spleen congestion; skull haemorrhage; worms in airsacs	Lung congestion and oedema and small random foci of necrosis in parabronchi; multiple small foci of necrosis in myocardium; congestion and random small foci of tubule necrosis in kidney cortex; brain and liver showed congestion; spleen had mild lymphoid necrosis	-	3+	*10	-	3+	*10
25. D-07-2372 Common Kestrel	2.3.4 /7	2+	Lung, spleen, brain congestion; skull haemorrhage; 1mm pale foci in kidney	Lung congestion and oedema; spleen, heart and brain were congested; kidney congestion and mild lymphohistiocytic interstitial nephritis; liver congestion, diffuse fatty degeneration and haemosiderosis	3+	-	*11	3+	2+	*11
26. D-07-2433 Scaly-breasted Munia	2.3.4 /3	3+	No changes evident	Lung congestion and oedema; brain and kidney congestion	1+	2+	*12	3+	3+	*12
27. D-07-2442 Chestnut Munia	2.3.4 /3	4+	No changes evident	Lung congestion and oedema; brain and kidney congestion	2+	2+	*13	3+	3+	*13
28. D-07-2572 Scaly-breasted Munia	2.3.4 /7	4+	Lung and brain congestion	Focal congestion in the kidney and congestion in the heart and brain	2+	2+	*14	2+	+/-	*14
29. D-07-2762 Long-tailed Shrike	2.3.4 /2	1+	Lung congestion and oedema; pharynx moist; skull and brain congested	Lung congestion and oedema; brain, heart, muscle and kidney congestion; also nematode parasites in renal cortex; tracheitis with epithelial and mucous gland loss	4+	4+	*15	3+	3+	*15
30. D-07-4925 Red-billed Starling	2.3.2	2+	Lung congestion and oedema	Lung had congestion and some oedema with small foci of peribronchial lymphoid necrosis; heart had multiple small foci of acute myocardial necrosis; kidney had congestion and multiple small foci of tubular necrosis; brain, liver and spleen showed congestion	-	-	*16	2+	2+	*16
31. D-07-5052 Common Magpie	2.3.2	1+	Haemorrhage in pectoral skin and muscle; lung oedema, congested spleen	Lung congestion and oedema; liver spleen and kidney showed congestion and multiple small foci of necrosis; heart had multiple small foci of acute myocardial necrosis; brain had mild congestion and pectoral muscle had haemorrhages and skin had haemorrhage and some dermal necrosis	3+	1+	*17	3+	1+	*17
32. D-07-5288 House Crow	2.3.2	2+	Lung congestion and oedema with some haemorrhage, trachea, intestines, pancreas congested; haemorrhage on right I cranium	Lung congestion and severe oedema with multiple haemorrhages; brain congestion and moderate multifocal vasculitis, gliosis and perivascular lymphohistiocytic cuffing; heart had moderate multifocal myocardial necrosis, lymphohistiocytic infiltrates and mineralisation; liver was congested and had mild multifocal hepatocyte necrosis with lymphohistiocytic and heterophil infiltrates and some haemosiderosis; kidney and proventriculus showed congestion	3+	1+	*18	3+	2+	*18

H5N1 grouping was based on phylogenetic analysis of HA gene sequences. Only one cluster was present in Clade 2.3.2 and 7 clusters were detected in Clade 2.3.4 (see Guan and Peiris et al., submitted for publication).

* Cryostat sections of Brain (B) and lung (L) were routinely examined by immunoperoxidase staining using monoclonal antibodies to H5 HA or avian influenza NP proteins. Results of other tissues (O) examined are shown below:

- *1 Liver: H5 2+, NP 1+; Pancreas: H5 2+, NP 2+; Spleen: H5 1+, NP 2+
- *2 Heart, liver, spleen: H5 1+, NP 1+
- *3 Heart: H5 1+, NP 1+
- *4 Heart, spleen, kidney, small intestine: H5 1+, NP 1+; Liver: H5 +/-, NP 1+
- *5 Heart: H5 -, NP 1+; Trachea: H5 +/-, NP +/-
- *6 Heart: H5 3+, NP 3+; Pancreas: H5 4+, NP 4+
- *7 Spleen: H5 2+, NP 2+
- *8 Spleen: H5 +/-, NP 1+
- *9 Heart, Kidney: H5 3+, NP 3+; small intestine: H5 2+, NP 3+
- *10 Bird 1 - Heart: H5 -, NP -; Bird 2 - Heart: H5 2+, NP 2+
- *11 Heart, Kidney: H5 1+, NP 2+; Pancreas: H5 nt, NP 3+
- *12 Heart: H5 2+, NP 3+
- *13 Heart: H5 -, NP -
- *14 Heart: H5 1+, NP 1+; Liver: H5 2+, NP 2+
- *15 Heart: H5 3+, NP 2+; kidney: H5 4+, NP 3+
- *16 Heart: H5 -, NP 3+; Kidney: H5 1+, NP 2+
- *17 Heart, kidney: H5 3+, NP 3+
- *18 Heart: H5 +/-, NP +/-; Kidney: H5 1+, NP 1+; Pancreas: H5 3+, NP 2+

nt – not tested.

Table 3

Virus isolation and PCR results for the H5N1 avian influenza virus infected wild birds detected in Hong Kong in 2006–2007

Viruses isolated	Species of bird	H5N1 Clade / cluster	Samples tested H5 Positive in VI and PCR *	VI #	H5 PCR	M PCR	N1 PCR	AF H5 PCR	AF M PCR	AF N1 PCR
1. D-06-0075	Magpie Robin	2.3.4 /6	C&T, B&L, I	+	+	+	+	+	+	+
2. D-06-0366	Magpie Robin	2.3.4 /6	T,L,B,Li,S,K,H,M	+	+	+	+	+	+	+
3. D-06-0540	Crested Myna	2.3.4 /5	C&T, L, B	+	+	+	+	+	+	+
4. D-06-0645	Common Magpie	2.3.4 /1	C&T, L, B	+	+	+	+	+	+	+
5. D-06-0718	Little Egret	2.3.4 /6	C&T, L, B	+	B +	B +	B +			
6. D-06-1038	Japanese White Eye	2.3.4 /4	T, B, I	+	+	+	+	+	+	+
7. D-06-2125	Common Magpie	2.3.4 /4	T,L,B,Li,S,K,H,P	+	C,T+	C,T+	C,T+			
8. D-06-2256	Common Magpie	2.3.4 /4	T,L,B,I	+	+	+	+	+	+	+
9. D-06-2454	Munia	2.3.4 /4	L,B	+	+	+	+	+	+	+
10. D-06-2469	White-backed Munia	2.3.4 /4	T,L,B	+	+	+	+	+	+	+
11. D-06-2512	Large-billed Crow	2.3.4 /4	C&T, L, B	+	+	+	+	+	+	+
12. D-06-2648	House Crow	2.3.4 /4	T,L,B,Li,S,K,H,P	+	B,L,T,I +	B,L,T,I +	B,L,T,I +			
13. D-06-2858	House Crow	2.3.4 /4	C,T,L,B,S	+	+	+	+			
14. D-06-3033	Common Magpie	2.3.4 /4	C&T,L,B,S,I	+	+	+	+	+	+	+
15. D-06-5211	Peregrine Falcon	2.3.2	C&T,L,B,S,K,H	+	+	+	+	+	+	+
16. D-07-0045	Scaly-breasted Munia	2.3.4 /2	C&T,T	+	+	+	+			
17. D-07-0458	Crested Goshawk	2.3.4 /2	C&T,L,B,S,I	+	+	+	+			
18. D-07-0719	House Crow	2.3.4 /7	C&T,L,S	+	C&T S +	C&T +	C&T S +	+	+	+
19. D-07-0737	Japanese White Eye	2.3.4 /7	C&T,L	+	T,L +		T,L +			
20. D-07-0828	White-backed Munia	2.3.4 /7	C&T,L,B,Li,I,S	+	+	+	+	+	+	+
21. D-07-1143	Peregrine Falcon	2.3.2	C&T,L,B,H,P	+	+	+	+			
22. D-07-1203	House Crow	2.3.4 /7	C&T	+	+	+	+	+	+	+
23. D-07-1993	Blue Magpie	2.3.4 /7	C&T,L,B,H,K,Li	+	-	+	C&T B,H+	-	+	+
24. D-07-2065	Silver-eared mesia #1	2.3.4 /7	C&T,L&H&T,B, K&S,I&Lj	+	B,L,T,H+	+	+	+/-B	+	+

Viruses isolated	Species of bird	H5N1 Clade / cluster	Samples tested H5 Positive in VI and PCR *	VI #	H5 PCR	M PCR	N1 PCR	AF H5 PCR	AF M PCR	AF N1 PCR
24. D-07-2065	Silver-eared mesia #2	2.3.4 /7	C&T, L&H&T, B, S, I	+	B, L, L K, S, +	+	+	+/-B	+	+
25. D-07-2372	Common Kestrel	2.3.4 /7	C&T, L, B, H, K, P, S	+	-	+	+	-	+	+
26. D-07-2433	Scaly-breasted Munia	2.3.4 /3	C&T, L, B	+	+	+	+	+	+	+
27. D-07-2442	Chestnut Munia	2.3.4 /3	C&T, T, I	+	+	+	+	+	+	+
28. D-07-2572	Scaly-breasted Munia	2.3.4 /7	C&T, L, B, H, I&L&K&S	+	C&T B, I, +	+	+	+/-B, L, H, I	+	+
29. D-07-2762	Long-tailed shrike	2.3.4 /2	C&T, L, B, H, K	+	+	+	+	+	+	+
30. D-07-4925	Red-billed Starling	2.3.2	C&T, L, K, S	+	+	+	+	+	+	+
31. D-07-5052	Common Magpie	2.3.2	C&T, L, B	+	+	+	+	+	+	+
32. D-07-5288	House Crow	2.3.2	C&T, L, B, K	+	+	L, B, K +	+	+	+	+

* C cloacal swab, T tracheal swab, L lung, B brain, H heart, S spleen, K kidney, Li liver, P pancreas, I intestines; link by & indicates pooled samples tested

VI virus isolation in chicken embryos; H5 PCR is RRT-PCR for H5 gene; M PCR is RRT-PCR for Matrix gene; N1 PCR is conventional RT-PCR for N1 neuraminidase gene; AF allantoic fluid harvest; Scored as (+) positive if all tissues tested were positive; (+/-) for the AF H5 PCR results indicates the tissues that were only positive using the CSIRO H5 primer/probe set; (-) negative; blank space indicates not tested by this test.

Table 4

Comparison of viral titres, H5 HA and M gene detection limits by RRT-PCR and antigen detection tests on the pooled cloacal and tracheal (C&T) swabs from 12 H5N1 virus infected wild birds in Hong Kong in 2006-2007.

Virus	Species of bird	H5N1 Clade / cluster *	C&T swab Virus Titer #	H5 RRT-PCR Detection limit ^	M RRT-PCR detection limit ^	NP Antigen Detection D	R
D-06-0366	Magpie Robin	2.3.4/6	4.5	1:10 ³	1:10	3+	1+
D-06-0540	Crested Myna	2.3.4/5	2.0	1:10 ³	1:10	1+	+/-
D-06-0645	Common Magpie	2.3.4/1	2.0	1:10 ³	1:10	2+	1+
D-06-2125	Common Magpie	2.3.4/4	6.3	1:10 ⁴	1:10 ²	1+	1+
D-06-5211	Peregrine Falcon	2.3.2	3.3	1:10	undiluted	+/-	-
D-07-0458	Crested Goshawk	2.3.4/2	1.5	1:10 ³	1:10	2+	1+
D-07-1143	Peregrine Falcon	2.3.2	2.5	1:10 ²	undiluted	+/-	-
D-07-1203	House Crow	2.3.4/7	5.3	1:10 ³	undiluted	+/-	-
D-07-2433	Scaly-breasted Munia	2.3.4/3	1.0	1:10 ³	undiluted	2+	1+
D-07-4925	Red-billed Starling	2.3.2	1.0	1:10	undiluted	-	-
D-07-5052	Common Magpie	2.3.2	2.5	1:10 ³	undiluted	3+	+/-
D-07-5288	House Crow	2.3.2	2.5	1: 10 ⁵	undiluted	2+	-

* H5N1 grouping was based on phylogenetic analysis of HA gene sequences. Only one cluster was present in Clade 2.3.2 and 7 clusters were detected in Clade 2.3.4 (see Guan and Peiris et al., submitted for publication).

Virus titer expressed as Log10 ELD50/0.1ml.

^ Detection limit by RRT-PCR for 12 viruses is shown as the highest serial 10-fold dilution to give positive RRT-PCR result using the Eurasian primers for H5-HA gene and SEPRL primers for M gene.

D = Directigen Flu A antigen detection test; R= Rocheby Avian influenza virus antigen test.